Phenobarbital induction of cytochromes *P*-450

High-level long-term responsiveness of primary rat hepatocyte cultures to drug induction, and glucocorticoid dependence of the phenobarbital response

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The induction of hepatic cytochromes *P*-450 by phenobarbital (PB) was studied in rat hepatocytes cultured for up to 5 weeks on Vitrogen-coated plates in serum-free modified Chee's medium then exposed to PB (0.75 mM) for an additional 4 days. Immunoblotting analysis indicated that *P*-450 forms PB4 (IIB1) and PB5 (IIB2) were induced dramatically (> 50-fold increase), up to levels nearly as high as those achieved in PB-induced rat liver *in vivo*. The newly synthesized cytochrome *P*-450 was enzymically active, as shown by the major induction of the *P*-450 PB4-dependent steroid 16 β -hydroxylase and pentoxeresorufin *O*-dealkylase activities in the PB-induced hepatocyte microsomes (up to 90-fold increase). PB induction of these *P*-450s was markedly enhanced by the presence of dexamethasone (50 nM–1 μ M), which alone was not an affective inducing agent, and was inhibited by > 90% by 10% fetal bovine serum. The PB response was also inhibited (> 85%) by growth hormone (250 ng/ml), indicating that this hormone probably acts directly on the hepatocyte when it antagonizes the induction of *P*-450 PB4 in intact rats. In untreated hepatocytes, *P*-450 RLM2 (IIA2), *P*-450 3 (IIA1) and NADPH *P*-450 reductase levels were substantially maintained in the cultures for 10–20 days. The latter two enzymes were also inducible by PB to an extent (3–4-fold elevation) that is comparable with that observed in the liver *in vivo*. Moreover, *P*-450c (IA1) and *P*-450 3 (IIA1) were highly inducible by 3-methylcholanthrene (5 μ M; 48 h exposure) even after 3 weeks in culture. In contrast, the male-specific pituitary-regulated *P*-450 form 2c (IIC11) was rapidly lost upon culturing the hepatocytes, suggesting that supplementation of appropriate hormonal factors may be necessary for its expression. The present hepatocyte culture system exhibits a responsiveness to drug inducers that is qualitatively and quantitatively comparable with that observed *in vivo*, and should prove valuable for more detailed investigations of the molecular and mechanistic basis of the response to PB and its modulation by endogenous hormones.

INTRODUCTION

Primary hepatocyte cultures have been useful for studying the mechanisms by which polycyclic hydrocarbons and glucocorticoids induce *de novo* synthesis of cytochromes *P*-450 (*P*-450) and other hepatic enzymes involved in xenobiotic metabolism [1–3]. The induction of specific *P*-450 enzymes by phenobarbital (PB), however, has been difficult to achieve in rodent and other mammalian hepatocyte systems. Evidence has accumulated in recent years to suggest that the absence of a strong PB response in cultured hepatocytes may reflect the de-differentiation of hepatocytes in culture, with a loss of liver-specific functions. Consistent with this hypothesis, rat hepatocytes have recently been reported to exhibit an improved response to PB and other mono-oxygenase inducers when placed in suitable medium [4] or when cultured on a reconstituted, non-specific, basement membrane matrix (Matrigel) under conditions that result in the maintenance of normal hepatocyte morphology and other liver-specific functions [5]. Normal hepatocyte cellular and biochemical properties can also be maintained by plating the cells at high density on hydrated rat-tail collagen [6]. Studies carried out in this laboratory have recently shown that, by use of Chee's essential medium, rat hepatocytes maintained on a collagen-based substrate (Vitrogen) exhibit long-term viability (20–30 days in culture) [7,8] with retention of several *P*-450-dependent

drug-metabolizing activities (D. J. Waxman & H. O. Jauregui, unpublished work). However, the absolute level of PB-induced *P*-450 activity in these cells corresponded to only about 10% of the level present in PB-induced rat liver. Similarly, in the case of hepatocytes cultured on Matrigel, the induced level of immunoreactive *P*-450 form PB4 (IIB1) (for explanation of *P*-450 nomenclature, see Abbreviations footnote), the major PB-inducible *P*-450 in rat liver, and of its mRNA, is up to 10-fold lower than is present in PB-induced rat liver *in vivo* [5]. In the present study we report that the levels of enzymically active *P*-450 PB4 protein that are inducible by PB *in vivo* can be attained in rat hepatocytes cultured on Vitrogen-coated plates in a modified Chee's medium (MCM) after treatment with PB. PB induction of *P*-450 PB5 (IIB2), *P*-450 3 (IIA1) and *P*-450 reductase is also shown to occur in these cells to an extent that is similar to that observed in intact rat liver, indicating that rat hepatocytes cultured under the conditions described in this study may be useful for studying the molecular basis of the PB response.

MATERIALS AND METHODS

Hepatocyte isolation and attachment to Vitrogen-coated plates

Hepatocytes were isolated from 150–250 g adult male Wistar rats (Charles River, Wilmington, MA, U.S.A.) by the collagenase

Abbreviations used: *P*-450, cytochrome *P*-450; PB, phenobarbital; MCM, modified Chee's medium; Dex, dexamethasone; 3MC, 3-methylcholanthrene. The two major PB-inducible rat hepatic *P*-450s are designated PB4 and PB5 [9] and correspond to rat *P*-450 gene products IIB1 and IIB2 respectively [10]. Other common designations of these two *P*-450 forms include PB4 = b or PB-B and PB5 = e or PB-D [11,12]. Other *P*-450 forms included in the present study are designated c (IA1), 3 (IIA1), RLM2 (IIA2), 2c (IIC11), PB-2a (IIIA1) and 2a (IIIA2). See [13] for a listing of alternative rat *P*-450 protein nomenclatures and [10] for an updated listing of the systematic designations of rat *P*-450 gene products (indicated by Roman numerals).

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Table 1. Composition of modified Chee's medium (MCM)

Numbers in parentheses refer to g/l or mg/l, as specified in the Table. The medium was purchased from Gibco in the form of a powder (formula no. 88-5046). As supplied, the medium does not include NaHCO_3 , L-arginine-HCl, L-glutamine, thymidine, insulin or gentamycin sulphate (items marked by *), which must be added in the amounts listed in the Table. For the experiments described in this report, the medium was supplemented with Dex (1 μM), transferrin (6.25 $\mu\text{g/ml}$) and selenium (6.25 ng/ml), except where indicated. The final pH of the complete medium was adjusted to 7.3 by addition of 10 M-NaOH and was found to be unchanged after a 48 h incubation with the hepatocytes in culture.

(1) Buffers and salts	
g/l:	Hepes (5.0), NaHCO_3 (2.2)*, NaCl (4.5), KCl (0.4), CaCl_2 anhydrous (0.2), MgSO_4 anhydrous (0.174), $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (0.125)
mg/l:	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.3), $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ (1.2), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (0.021), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.015), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.86)
(2) Amino acids	
g/l:	L-Arg.HCl (0.168)*, L-Ala (0.072), L-Asn, H_2O (0.12), L-Asp (0.107), L-cystine, 2HCl (0.0626), L-Glu (0.118), L-Gln (0.584)*, Gly (0.06), L-His, HCl, H_2O (0.084), L-Ile (0.2196), L-Leu (0.4392), L-Lys.HCl (0.2824), L-Met (0.06), L-Phe (0.132), L-Pro (0.092), L-Ser (0.084), L-Thr (0.1904), L-Trp (0.032), L-Tyr, $2\text{Na}_2\text{H}_2\text{O}$ (1.038), L-Val (0.3744)
(3) Vitamins	
mg/l:	Ascorbic acid (25), D-biotin (0.03), nicotinic acid (4), D-Ca-pantothenate (4), pyridoxal.HCl (4), thiamine.HCl (4), folic acid (4), riboflavin (0.4), vitamin B_{12} (0.003)
(4) Other	
g/l:	Dextrose (4.5), D-sorbitol (0.1), β -sodium glycerophosphate (1.0), D-gluconolactone (0.178), oxaloacetic acid (0.265)
mg/l:	Deoxycytidine.HCl (12.77), thymidine (10)*, protamine sulphate (2), choline chloride (4), i-inositol (7), insulin (6.25)*, gentamycin sulphate (50)*.

(Worthington Biochemical) method of Seglen [14], with pre-perfusion and perfusion steps done *in situ* rather than after removal of the liver. Typical yields were $(350-450) \times 10^6$ cells/g of liver, with a viability of $93 \pm 3\%$ as assessed by Trypan Blue exclusion and lactate dehydrogenase assays [15]. The final cell pellet was resuspended in 25 ml of suspension buffer [14] containing insulin (10 munits/ml) and dexamethasone (Dex) (1 μM) and kept at 4 °C until ready for use (up 1 h). Cells were then suspended at 4×10^6 cell/5 ml of MCM, which is based on Chee's essential medium [7,16] modified as in Table 1. In addition, 1 μM -dexamethasone (Dex), transferrin (6.25 $\mu\text{g/ml}$) and selenium (6.25 ng/ml) were added, unless indicated otherwise. The cells were seeded on to 60 mm Nunc Permanox tissue culture dishes (dish no. 174888; Nunc Inc., Naperville, IL, U.S.A.) precoated with Vitrogen (Collagen Corp., Palo Alto, CA, U.S.A.) as described below. Results obtained with Nunc Permanox dishes were superior to those obtained with several other culture dishes, including Corning and Primaria. Hepatocytes were allowed to attach at 37 °C in an atmosphere of air/ CO_2 (19:1) and a relative humidity of 98%. After 3 h, the medium with unattached cells was aspirated and fresh MCM was added to the cultures. Previous studies have established that Chee's essential medium has a unique ability to preserve the protein content of adult rat hepatocytes in long-term culture [7]. The medium is based on Eagle's minimum essential medium modified by the addition of large amounts of select amino acids, salts and vitamins, with an increased buffering capacity afforded by the inclusion of Hepes (5g/l) and sodium bicarbonate (2.2 g/l) (Table 1):

Tissue culture dishes were coated with Vitrogen using morpho

carbodi-imide (Aldrich, no. C10,640-2) as coupling agent [17]. Vitrogen was diluted to 90–100 $\mu\text{g/ml}$ in an aqueous solution of morpho carbodi-imide (130 $\mu\text{g/ml}$, final concentration), and the resulting solution was coated on the tissue culture plates (2 ml/60 mm plate), which were incubated at 37 °C overnight. Unbound collagen was then removed by aspiration, and the plates were rinsed twice with 0.9% NaCl and stored at 4 °C until ready for use.

Culturing of primary hepatocytes and drug induction

Primary hepatocyte cultures, prepared as described above, were incubated at 37 °C in an atmosphere of air/ CO_2 (19:1) and a relative humidity of 98%, with addition of fresh MCM at 48 h intervals. Cells were induced with PB (0.75 mM), which was added to the medium just after cell attachment ('continuous PB') or for 96 h before harvesting the cells ('96 h PB'). Under these conditions, PB in the medium was shown to be stable for at least 48 h. In other experiments cells were induced with 3-methyl-cholanthrene (3MC) (5 μM) added to the medium 48 h before harvesting the cells. The inhibitory effect of growth hormone on the PB response was examined by adding 250 ng of human growth hormone/ml (2.4 units/mg; National Hormone and Pituitary Program, University of Maryland, Baltimore, MD, U.S.A.) to the medium 24 h before PB addition, i.e. 120 h before harvesting the cells. In some experiments the medium was supplemented with 10% fetal bovine serum (Gibco); in this latter instance, transferrin and selenium were excluded. In other experiments the Dex concentration of the medium was decreased to 0 or 50 nM, as indicated.

Isolation of hepatocyte microsomes and analysis of P-450 proteins and activities

Cells were harvested from the tissue culture dishes by scraping, recovered by sedimentation and stored at -80 °C until ready for analysis. Frozen cell pellets were homogenized in 0.1 M-potassium phosphate, pH 7.4, 1 mM-EDTA, 20% glycerol [$(3-7) \times 10^6$ cells/ml of buffer and $(20-32) \times 10^6$ cells/treatment group (5-7 dishes)] with a Dounce manual homogenizer (40 strokes/sample). Particulate matter was removed by centrifugation for 7 min at 12000 g and a microsomal fraction was then pelleted by centrifugation for 60-75 min at 100000 g. Microsomal pellets were resuspended in homogenization buffer and stored in aliquots at -80 °C. Protein was assayed using the Bradford protein assay reagent (Bio-Rad) with bovine serum albumin as standard.

Steroid hydroxylase activities were determined by incubation of isolated microsomes (40-60 μg of protein) in 0.4 ml of 0.1 M-Hepes buffer, pH 7.4, containing 50 μM -[4- ^{14}C]androstenedione or [4- ^{14}C]testosterone (5 mCi/mmol; Amersham) and NADPH (1 mM) for 10-15 min at 37 °C using analytical methods detailed previously [18]. Results presented are representative of a single set of steroid hydroxylase assays whose results were verified using the second steroid substrate. In addition, results were generally confirmed by parallel analyses of at least one other batch of cells cultured for a different length of time. Relative rates of hepatocyte microsomal pentoxoresorufin O-dealkylase activity [19] and P-450 reductase activity (monitored by cytochrome c reduction) [20] were assayed in duplicate or triplicate.

For Western blot analysis of individual microsomal P-450s, isolated hepatocyte microsomes were electrophoresed on SDS polyacrylamide gels, transferred to nitrocellulose and then immunoblotted with anti-P-450 antibodies (see below) using an alkaline phosphate-conjugated second antibody for detection [20]. Laser densitometry and P-450 immunquantifications were carried out as described [20]. The following antibodies were used: antibodies to P-450 PB4 (IIB1) that cross-react with P-450 (IIB2) [21]; antibodies to P-450 3 (IIA1) that cross-react with P-

450 RLM2 (IIA2) [20]; monoclonal antibody 1-7-1, which is reactive with both *P*-450c (IA1) and *P*-450d (IA2) (as well as with an unidentified microsomal polypeptide under the conditions used for Western blotting in these analyses [20]), which was kindly provided by Dr. S. Park and Dr. H. Gelboin, National Cancer Institute, Bethesda, MD, U.S.A. [22]; and antibody to *P*-450 PB2a (IIIA1) which cross-reacts with *P*-450 2a (IIIA2) [20]. In each case, except for that of *P*-450 PB2a and *P*-450 2a, the pairs of cross-reactive *P*-450 antigens could be visualized individually on the Western blots as a result of their electrophoretic separation on the SDS gels.

PB-induced rat liver microsomes

P-450 activities and protein levels determined for hepatocytes induced with PB in culture were compared with those determined in parallel incubations of microsomes prepared from adult male rat liver induced with PB *in vivo* [9]. Liver microsomes prepared from a total tissue homogenate as outlined elsewhere [20] or from freshly isolated hepatocytes prepared by collagenase perfusion as described above yielded similar *P*-450 protein and activity levels.

RESULTS

Untreated adult male rat hepatocytes isolated by collagenase perfusion were cultured on Vitrogen-coated plates in MCM + 10 % fetal bovine serum for up to 7 days in the presence or absence of 0.75 mM-PB. The concentration of PB used in these studies was chosen as optimal with respect to induction of hepatocyte *P*-450 diazepam-metabolizing activities on the basis of preliminary experiments carried out from 0.5–2 mM-PB (results not shown). Exposure to PB was carried out either continuously or for 96 h before harvesting the cells. Hepatocyte microsomes were prepared and *P*-450 PB4-dependent pentoxyresorufin *O*-dealkylase activity [19] was then determined. PB was thus shown to induce microsomal *O*-dealkylase activity up to ~20-fold compared with the PB-deficient controls (Fig. 1). Similar results were obtained when *P*-450 PB4-dependent [13] steroid 16 β -hydroxylase activity was monitored (androstenedione 16 β -hydroxylation rates of 0.07, 0.47 and 0.59 nmol/min per mg res-

Fig. 1. Microsomal pentoxyresorufin *O*-dealkylase activity in PB-treated primary adult rat hepatocytes

Hepatocytes were cultured in MCM containing 10 % fetal bovine serum for 0–7 days. Cells were not induced (■—■) or were induced with 0.75 mM-PB for 96 h before harvesting the cells on day 7 (x) or with PB by continuous exposure (■---■). Pentoxyresorufin metabolism activity in isolated hepatocyte microsomes is expressed in arbitrary fluorescent units (f.u.) (average of duplicate determinations) and can be compared with that measured in parallel for liver microsomes prepared from PB-induced adult male rat liver (1150 f.u./min per mg).

Table 2. PB induction of steroid 16 β -hydroxylation in cultured hepatocytes: influence of the presence of serum, Dex and growth hormone

Androstenedione 16 β -hydroxylase activity was assayed in hepatocyte microsomes prepared from 10-day-old cells that either were not treated with PB (UT) or were induced with PB (cont = continuous) or 3MC as indicated. Culture conditions were varied with respect to the presence or absence of 10 % fetal bovine serum or Dex (1 μ M) in the medium or by addition of growth hormone (GH, 250 ng/ml) 24 h before PB treatment. Values in parentheses indicate fold increases in activity compared with the uninduced control

Expt. 1	Androstenedione 16 β -hydroxylase activity (nmol/min per mg of microsomes)			
	UT	PB (96 h)	PB (cont)	3MC (48 h)
+ Serum, + Dex	0.04	0.28 (7)*	0.45 (11)	0.02 (0.5)
– Serum, + Dex	0.12	5.92 (49)	4.02 (34)	0.13 (1.1)
Liver microsomes*	0.24	5.39 (22)	–	–

Expt 2	PB			
	UT	PB (96 h)	(96 h)+GH	UT+GH
– Serum, – Dex	0.02	0.40 (20)	0.04 [10%]†	0.02
– Serum, + Dex	0.10	8.70 (87)	1.14 [13%]†	0.03

* Activities measured for either uninduced or PB-induced rat liver microsomes.

† Activities expressed as a percentage of the PB (96 h) group incubated without growth hormone.

pectively determined for uninduced, continuous-PB-induced and 96 h-PB-induced hepatocyte microsomes prepared from day 7 cells).

Although a significant induction of these *P*-450 PB4-dependent activities was thus obtained, the specific activity for day 7 continuous-PB-induced cells was only about 6 % of that exhibited by microsomes isolated from PB-induced rat liver (cf. pentoxyresorufin *O*-de-ethylase activity of 1150 fluorescence units/min per mg and androstenedione 16 β -hydroxylase activity of 8.7 nmol/min per mg). The culture conditions were therefore modified in an attempt to enhance the induction of these *P*-450 activities. It was thus determined that when the cells were cultured in the same medium, but in the absence of serum, *P*-450 PB4-dependent steroid 16 β -hydroxylase activity was induced to a level equivalent to that achieved in the liver *in vivo*, which under these circumstances corresponded to a 50–90-fold induction (Table 2). In a control experiment, hepatocyte microsomal steroid 16 β -hydroxylase activity was not increased by the *P*-450c (IA1) inducer 3MC. The PB response in these cells was markedly enhanced by the presence of Dex, which by itself had a negligible effect on the PB-inducible *P*-450 activity (Table 2, expt. 2; Table 3). Elimination of transferrin and selenium from the medium did not impair the PB response (Table 3), whereas the addition of growth hormone was found to be markedly inhibitory (> 85 % inhibition at 250 ng/ml; Table 2). Growth hormone has been reported to antagonize the PB induction of *P*-450 *in vivo* [23], and may be one of the components in serum responsible for its inhibition of the PB response.

The long-term viability of primary rat hepatocytes can be enhanced by Chee's essential medium [7]. An examination of the long-term responsiveness of the hepatocytes to PB using MCM and the culture conditions described in this paper revealed that the cells were highly inducible for about 20 days, with the steroid 16 β -hydroxylase activity of the induced cells (4.0–6.2 nmol/min per mg of microsomal protein) (Fig. 2) being comparable with

Table 3. Dependence of the PB response on Dex

Adult rat hepatocytes were cultured for 10 days in MCM under standard conditions [UT, untreated with PB; PB (96 h), PB-induced for 96 h before harvesting the cells], except that the Dex concentration was varied, as indicated, and in one group transferrin and selenium were omitted (–TS). Androstenedione 16 β -hydroxylase activity was assayed in isolated hepatocyte microsomes.

Dex (μ M)	Androstenedione 16 β -hydroxylase activity (nmol/min per mg)	
	UT	PB (96 h)
0	0.03	1.17
0.05	0.33	4.36
1	0.28	4.26
1 (–TS)	0.18	4.59
Liver microsomes	0.39	6.17

Fig. 2. PB induction of microsomal androstenedione 16 β -hydroxylase activity in long-term hepatocyte cultures

Hepatocytes were cultured in MCM for 0–40 days and were either untreated (x) or were induced with PB by continuous exposure (●) or for 96 h before harvesting the cells (■), as indicated. Androstenedione 16 β -hydroxylase activity was assayed in isolated hepatocyte microsomes. Results shown are representative of multiple independent assays for 16 β -hydroxylation using either androstenedione or testosterone (results not shown) as substrate.

that determined for liver microsomes isolated from rats induced with PB *in vivo* (4.6–5.7 nmol/min per mg; Table 2; and results not shown). A significant PB response was also observed in cells maintained for 30 and 40 days in culture, although the induced activity was only 30–50% of that achieved in cells cultured for shorter periods of time (Fig. 2). When compared with a 96 h induction period, continuous PB exposure was less effective, particularly in cells cultured beyond 20 days.

Western blot analyses were then carried out to ascertain whether the observed induction of *P*-450-dependent steroid 16 β -hydroxylase activity in these cells was accompanied by an elevation of *P*-450 form PB4 (IIB1), which is the major catalyst of this activity in PB-induced rat liver microsomes [13]. Indeed, both *P*-450 PB4 and the closely related *P*-450 PB5 (IIB2) were highly induced in the hepatocytes, even after 40 days in culture

Fig. 3. Western blot analysis of *P*-450 PB4 and *P*-450 PB5 in PB-induced hepatocyte microsomes

Microsomes (2.5 μ g of protein/lane) prepared from uninduced (–) and 96 h-PB-induced (+) hepatocytes isolated after 20 (lanes 2 and 3), 30 (lanes 4 and 5) or 40 (lanes 6 and 7) days in culture (same samples as in Fig. 2) were analysed for *P*-450 forms PB4 and PB5 by Western blotting. Also shown is a sample of day 0 (uninduced) hepatocyte microsomes, which shows a minor cross-reactive band of lower apparent M_r than *P*-450 PB4 (lane 1), as well as a microsomal sample prepared from PB-induced rat liver (2.5 μ g, lane 8; 1 μ g, lane 9), which demonstrates that the specific content of microsomal *P*-450 PB4 and PB5 present in the induced cultured cells at the 20 day time point (lane 3) is nearly as high as that induced in rat liver *in vivo* (lane 8) (also see the text). Microsomal bands visualized on the Western blot were shown to co-migrate with purified *P*-450 PB4 and PB5 standards [9], as marked on the right.

(Fig. 3). The induction of these proteins was enhanced by Dex and was inhibited both by growth hormone and by 10% serum (results not shown), in accordance with the *P*-450 PB4-associated steroid 16 β -hydroxylase activity data described above (Tables 2 and 3). Quantitative analysis revealed that the levels of these two *P*-450 proteins in the induced hepatocyte microsomes (*P*-450 PB4 = 0.88 nmol/mg; *P*-450 PB5 = 0.31 nmol/mg of microsomal protein in 20-day-old cells) and their ratios (PB4/PB5 = 2.8) were similar to those measured in PB-induced rat liver microsomes (PB4 = 1.30 nmol/mg; PB5 = 0.73 nmol/mg; ratio = 1.8). Thus, under the culture conditions employed in this study, PB induces *P*-450s PB4 and PB5 in a manner and to an extent that is comparable with that which occurs in the liver *in vivo*.

Fig. 4. Influence of hepatocyte culture and drug inducers on hepatocyte microsomal testosterone (T) hydroxylase and *P*-450 reductase activities

Microsomes were isolated from hepatocytes cultured for 20 days in MCM in the absence of inducer (■) or in the presence of PB (0.75 mM for 96 h; ■) or 3MC (5 μ M for 48 h; ■), and then assayed for the indicated hydroxylase activities and for *P*-450 reductase activity (cytochrome *c* reduction). Activities are compared with those of freshly isolated hepatocytes (day 0 samples; ■). Shown are representative analyses determined in a single set of parallel analyses.

Fig. 5. Western blot analysis of *P*-450s 3 (IIA1) and RLM2 (IIA2) (A), family IIIA *P*-450(s) (b) and *P*-450c (IA1) (c) in cultured rat hepatocytes

Microsomes prepared from hepatocytes cultured as described in the legend to Fig. 4 were analysed for individual *P*-450s by Western blotting (a, 8 μ g of protein/lane; b, 5 μ g/lane; c, 20 μ g/lane). Time in culture was 20 days (a and b, lanes 1–4; c, lanes 3 and 4) or 10 days (c, lanes 1 and 2). Microsomes isolated from freshly isolated hepatocytes are shown for comparison [day 0 (0 d) samples]. —, No induction; PB, cells induced with PB for 96 h; PB', continuous PB induction in culture; MC, 3MC induction for 48 h; BNF μ S, microsomes isolated from livers of β -naphthoflavone-induced rat. In (a), band x represents a *P*-450 IIA-immunoreactive band that has not been identified [20], and the two immunoreactive *P*-450 IIIA proteins [*P*-450 2a (IIIA2) and *P*-450 PB2a (IIIA1)] are not resolved in the blot shown in (b). Also unidentified are bands X and Y of (c), which are expressed constitutively in day 0 cells (results not shown): in the case of band X, a significant induction by 3MC was noted (lanes 1 versus 2; 3 versus 4). *P*-450 c expression was not detectable in day 0 cells (results not shown). Results shown were confirmed in replicate analyses of these samples, as well as by Western blot analysis of hepatocyte microsomes isolated from *t* = 10-day cells (results not shown). Identification of the microsomal *P*-450s marked on the right of each panel was made on the basis of their co-migration with purified *P*-450 standards and was supported by the *P*-450 form specificities of the antibodies used in these analyses, as described previously [20–22].

[21] microsomal testosterone 2 α -hydroxylase (Fig. 4) and 16 α -hydroxylase activities (results not shown). Similar decreases were observed in cells cultured for as little as 5 days. This is not surprising, insofar as the expression of this *P*-450 is regulated *in vivo* by gonadal and pituitary hormones [13,24,25], which are absent from the culture medium. In contrast, the hepatocytes continued to express the male-specific *P*-450 RLM2 (IIA2) (Fig. 5a) [20] and its associated testosterone 15 α -hydroxylase activity (results not shown). This is consistent with the finding that *P*-450 RLM2 is expressed in adult rat liver even in the absence of pituitary hormones [20]. Other constitutively expressed *P*-450 forms, such as the testosterone 7 α -hydroxylase *P*-450 3 (IIA1) and the testosterone 6 β -hydroxylase *P*-450(s) of family IIIA, were maintained at ~35–40% of their initial levels in cells kept for up to 20 days in culture. Moreover, the expression of these latter *P*-450s and their associated steroid hydroxylase activities was inducible by PB by 3–6-fold (Figs. 4 and 5), as also occurs *in vivo* [11–13]. PB also induced, by ~4-fold, hepatocyte microsomal *P*-450 reductase, which plays an obligatory role in electron transfer from NADPH to each of the microsomal *P*-450 enzymes (Fig. 4). Finally, *P*-450 3 levels in these cells were also inducible by 3MC (5 μ M for 48 h) (Figs. 4 and 5), which is an effective inducer of this *P*-450 in rat liver *in vivo* [26]. 3MC also effected a dramatic induction of *P*-450c in the cultured cells, as well as a more modest induction of family IIIA *P*-450(s) (Fig. 5) and their associated steroid 6 β -hydroxylase activity (Fig. 4).

DISCUSSION

Molecular studies of the mechanism(s) by which PB induces individual hepatic cytochrome *P*-450 forms have been hampered by the absence of a cell culture system that consistently exhibits a strong pharmacological response to this drug. Although a significant PB induction of the major PB-inducible rat hepatic *P*-450 forms PB4 (IIB1) and PB5 (IIB2) has been reported in some culture systems [4,27], the absolute levels of the cytochromes that accumulate in these cells are very low compared with those achieved in PB-induced liver *in vivo*. The response to PB can be improved significantly when primary rat hepatocytes are cultured on the tumour-derived basement membrane matrix Matrigel [5]. However, the induced mRNA and apoprotein levels of *P*-450 PB4 in this system are still about 10-fold lower than in PB-induced rat liver; moreover, the extent to which enzymically active holo-cytochrome *P*-450 is formed by these cells is unknown. In contrast, the present report describes a hepatocyte culture system in which PB can induce high levels of enzymically active holo-cytochrome *P*-450 forms PB4 and PB5. This induction, as well as that of the 3MC-inducible *P*-450c (IA1), can be achieved for a full 5 weeks after the cells are placed in culture. Moreover, the prolonged viability of these hepatocytes and their long-term maintenance of the differentiated state with respect to the PB response does not require the use of Matrigel as a substrate.

Two other hepatic microsomal enzymes, *P*-450 3 (IIA1) and *P*-450 reductase, were also inducible by PB in the present culture system. In contrast to the > 50-fold increase in *P*-450s PB4 and PB5, *P*-450 3 and *P*-450 reductase were induced by PB by only 3–4-fold. Similar increases in these two proteins are observed in PB-treated rats *in vivo* (e.g. [11,12,24]), suggesting that the present culture conditions *in vitro* constitute a good model system for studying PB-responsive hepatic genes. A further indication of the suitability of this system for studying the PB response is provided by the observation that growth hormone can significantly inhibit the increase in *P*-450s PB4 and PB5, both

The influence of the present culture conditions on several other *P*-450 forms, including both constitutive and PB-inducible cytochromes, was next examined. Expression of the constitutive male-specific *P*-450 form 2c (IIC11) was decreased by > 80% in cells cultured for 20 days, as shown by the decline of its associated

in vivo [23] and in our culture system. This indicates that growth hormone probably acts directly on the hepatocyte to antagonize the induction of *P*-450 PB4 expression in PB-treated rats *in vivo*. The mechanistic basis for this inhibitory effect of growth hormone is presently unclear, but may relate to the suppressive effect that growth hormone can have on the expression of *P*-450j (IIE1) [28] as well as of several male-specific *P*-450 forms in adult rat liver [20,29,30].

Although readily apparent in freshly isolated adult male rat hepatocytes, expression of the male-specific *P*-450 form 2c (IIC11) was rapidly lost during the first week in culture. This observation is in accord with the findings of others using either Vitrogen-based hepatocyte cultures [31] or the Matrigel system [32], and suggests that supplementation of one or more pituitary-dependent hormones may be necessary to maintain this hormone-dependent cytochrome in culture. In contrast, expression of the male-specific *P*-450 RLM2 (IIA2) was maintained in the hepatocytes, in accord with our earlier demonstration that this *P*-450 is not obligatorily dependent on pituitary hormones for expression in rat liver [20]. Although immunoreactive *P*-450 2a (IIIA2), which is also adult-male-specific and hormonally regulated [20,24], was also maintained in the cultured cells, this may in part reflect an induction of the closely-related *P*-450 PB2a (IIIA1) by Dex present in the medium [3]; this latter *P*-450 cannot be resolved from *P*-450 2a on Western blots of the hepatocyte microsomes.

Although Dex alone was at best a poor inducer of *P*-450 PB4 in the cultured hepatocytes, it was highly synergistic with PB, and even low concentrations (50 nM) were sufficient to impart a high level of *P*-450 PB4 expression to the PB-treated cells. Dex has previously been reported to enhance the induction by PB of *P*-450-dependent aldrin epoxidase activity in cultured fetal rat hepatocytes [33]. Although Dex is a poor inducer of *P*-450 PB4 protein in rat liver *in vivo* [23,34], in mouse liver it is a strong inducer of the corresponding PB-inducible subfamily IIB *P*-450(s) [35]. The mechanistic basis for the synergistic interaction between PB and Dex described in the present study is currently unknown, but it may have analogy to the synergistic effect of Dex on the induction of *P*-450c by 3MC, which involves the participation of a glucocorticoid response element present within intron 1 of the *P*-450c gene [36]. Interestingly, a glucocorticoid-like response element is present in the 5'-flanking region of the *P*-450 PB5 gene [37]. It is possible, however, that the Dex effect observed in the present study is the result of an indirect effect of this glucocorticoid on the synthesis of other factors (e.g. extracellular matrix proteins) that may be required for maintenance of the hepatocytes in an appropriate differentiated state [5,6,38,39].

In summary, the present study demonstrates that a high level, long-term, and *in vivo*-like response to PB can be achieved in hepatocytes cultured on Vitrogen-coated plates without the addition of other extracellular matrix components such as Matrigel. This culture system should facilitate studies on the metabolism of carcinogens, anti-cancer drugs and other substrates of hepatic *P*-450s in an intact hepatocyte system, as well as mechanistic studies of the phenobarbital response and its modulation by endogenous factors such as growth hormone and glucocorticoids.

Note added in proof (received 13 August 1990)

In agreement with the present study, Sinclair and associates [40] have also observed that a good response to phenobarbital can be obtained in cultured rat hepatocytes without a need for Matrigel.

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